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(54) Title: METHOD AND KIT FOR PREDICTING THE THERAPEUTIC RESPONSE OF A DRUG AGAINST A MALIGNANT TUMOUR

## (57) Abstract

The present invention relates to a method of predicting the therapeutic response of a drug against a malignant tumour in a human patient, using a sample of a fine needle aspirate of the tumour from the patient. The method comprises the steps of identifying and estimating in the sample the number of cells belonging to one class of cells in relation to other cells in the sample, and comparing the estimation with a previously made analysis demonstrating correlation between the number of the cells and parameters of the therapeutic response of the drug directed towards the tumour in human patients, the results of the comparison being indicative of the therapeutic response of the drug in human patient. A diagnostic kit comprises a mixture of enzymes rendering the sample of the tumour a single cell suspension, a first mixture of monoclonal antibodies directed towards tumour associated antigens on tumour cells in the single cell suspension, said antibodies having a first label, and a second mixture of two types of monoclonal antibodies directed towards two specific antigens on lymphocytes, said antibodies having a second and a third label, respectively.

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**METHOD AND KIT FOR PREDICTING THE THERAPEUTIC  
RESPONSE OF A DRUG AGAINST A MALIGNANT TUMOUR.**

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The present invention relates generally to a method of predicting the therapeutic response of a drug. More specifically, the invention relates to a method of predicting the therapeutic response of a drug directed towards a malignant tumour in a human patient, and to a diagnostic kit in order to enable the method to be applied routinely

The importance of lymphocytes in the control by the immune system of malignant tumours as well as the necessary presence of these cells in tumours has been known for several decades. However, nobody has studied the presence of lymphocytes in tumours, using fine needle aspirates, in relation to immunotherapy and the effect of treating a disease with a successful outcome.

The outcome of a treatment of a patient with a specific drug against a malignant tumour is often unpredictable. Only about 15-20 % of the patients respond to the medication of each type of treatment. The patients receiving the medication are subjected to an unnecessary suffering since adverse reactions often are obtained from the drug used. Mostly, the effect of the drug is not shown before 3-6 months of treatment. A drug for treatment of a malignant tumour is often expensive and a considerable cost reduction would be obtained if the drug used is effective. It would therefore be of great importance if patients with a high probability to respond could be identified before the onset of treatment.

For example, interferon-alpha (IFN- $\alpha$ ) has a documented activity against malignant melanoma. Five to ten million units IFN- $\alpha$  three times weekly seems, at the cost of reasonable side effects, (Legha et al.) to be an optimal dose range. The treatment efficacy varies in different stu-

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dies (Creagan et al., Gundersen, Legha et al.) and the over-all response rate (CR+PR) to IFN- $\alpha$  alone is only about 20 % (Creagan et al., Legha et al.). It would therefore be a considerable improvement if patients with a high probability to respond to this treatment could be identified using predictive tests.

Besides having an antiproliferative activity (Balkwill et al., Riviere et al.), the antitumour effect of IFN- $\alpha$  can be due to modulation of tumour cells, e.g. induction of 2,5 -A synthetase or increased expression of cell surface proteins, MHC I and tumour associated antigens which are of importance for the immunological control of tumours. In addition, IFN- $\alpha$  modulates several immune functions (12 Friedman). There are still no firm data demonstrating which of these activities of IFN- $\alpha$  that is the most important one for the antitumour activity or if they all contribute.

As IFN- $\alpha$  modulates the activity of various cells in the immune system, e.g. activation of macrophages, NK-cells, cytotoxic T-cells and B-cell function (Friedman et al.), the therapeutic effect might depend on the immune status of the patients when IFN- $\alpha$  therapy is initiated.

Immune reactivity to malignant melanoma has been demonstrated as spontaneous regression (Mc Govern et al., Sondergaard et al., Ronan et al., Kang et al.), occurrence of antibodies of prognostic significance against melanoma associated antigens and specific cytotoxic lymphocytes (Abershold et al.). Conflicting results have, however, been reported on the prognostic significance of lymphocytic infiltration in primary malignant melanoma. Some studies found a significantly better prognosis if the primary lesions had a prominent infiltration of lymphocytes (Hansen et al., Larsen et al.) whereas others found no such correlation (Balch et al.) Three studies also found a relation between tumour thickness and lymphocytic infiltration.

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(Balch et al., Hansen et al., Larsen et al.). This discrepancy was further studied by Mc Govern et al. who found the lymphocytic infiltration at the base of the melanoma to be of prognostic significance in contrast to lymphocytic infiltration at the margins. These authors also found that the lymphocytic infiltration at the tumour base was reduced as tumour thickness increased. A reasonable strategy to treat malignant melanoma would therefore, based on the immune reactivity to this type of malignancy, be to enhance the anti-tumour immune reactivity by immunomodulating substances, such as interferons and Interleukin-2.

The purpose of the present invention is to produce a method of predicting the therapeutic response of a drug against a malignant tumour in a human patient, using a sample of a fine needle aspirate of said tumour from said patient. The method according to the invention is thus based on a direct diagnosis in the tumour of the accumulation of lymphocytes.

In order to achieve this purpose, the method according to the invention has been given the characterizing feature of claim 1.

The method of predicting the therapeutic response of a drug concerns patients with a regional as well as a generalised metastasis formation. It is also possible by means of the method according to the invention to predict and monitor immunotherapy.

#### MATERIAL AND METHODS

##### Patients data.

This report includes 41 patients with metastatic malignant melanoma, 23 males and 18 females. Median age was 60 years (range 33 to 77) and Karnowsky performance status was 70 or more. All patients had histologically proven primary malignant melanoma and recurrences were cytologically proven before start of treatment. Twenty patients had regional metastases (20 lymphnode metastases), which

in general were excised after 1 to 3 weeks of IFN- $\alpha$  treatment and 21 patients had systemic disease with the following metastatic sites: 2 cutaneous, 5 subcutaneous, 16 lymphnodes, 7 pulmonary, 3 bone, and 16 visceral (9 liver, 1 ovarian, 1 pancreatic, 1 vulvar, 1 adrenal gland and 3 spleen metastases). The number of metastatic sites were 1 in 7 patients, 2 in 5 patients, 3 in 4 patients, 4 in 1 patient and 5 or more in 4 patients. No patient had previously been treated except for surgical removal of primary lesions or metastases. Patients with symptoms of brain metastases were not included in this study.

#### Patients evaluation

Pre-treatment investigations included ECG, abdominal CT/US, chest X-ray, bone scintigraphy and blood samples for measurements of creatinine, bilirubin, alkaline phosphatase, alanine-aminotransferase, lactat-dehydrogenase, alpha amylase, haemoglobin, white blood cells and thrombocytes.

#### Treatment schedule

In a pilot study including 12 patients (4 with regional disease and 8 with general metastases), IFN- $\alpha$  was given s.c. three days weekly at a dose of  $10^6$  IU. In this pilot study it was decided to give Cyclophosphamide and Indomethacin in addition to IFN- $\alpha$  in an attempt to enhance the therapeutic efficacy of IFN- $\alpha$ . Patients were treated with cyclophosphamide at a dose of  $300\text{mg}/\text{m}^2$  administered as i.v. bolus every three weeks and 6 of these patients were also treated with 50 mg of Indomethacin three times daily and 4 of the 6 was in addition given 400mg of Cimetidin twice daily. Tumour response was evaluated in patients with regional resectable disease after one to three weeks and in patients with distant metastases after one to three treatment cycles, one cycle being three weeks.

The pilot study was followed by a confirmatory study including 29 patients (16 with regional resectable disease and 13 with systemic disease) patients were treated with

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IFN- $\alpha$  alone, sc. at a dose of 10 milj. IU three times weekly in order to demonstrate the predictive value of CD4+ tumour infiltrating lymphocytes for the outcome of IFN- $\alpha$  treatment. In patients with regional resectable disease the treatment was given during one to three weeks before surgery and in the group with distant metastases treatment was given 3 weeks followed by one week without treatment. No other treatments were added in either group in the confirmatory study. Tumour response was evaluated in patients with regional resectable metastases after the first cycle and in patients with distant metastases after one to three treatment cycles.

As no differences were found between the two studies regarding response rates or histopathological regression of tumour infiltrating inflammatory cells the results of them were added for the final analyses.

#### Monoclonal antibodies.

CD4 (Leu-3a, Becton-Dickinson): The antigen is present on the helper T subset and in low density on monocytes and in the cytoplasm of monocytes and macrophages.

CD8 (Leu-2a, Becton-Dickinson): The antigen is present on cytotoxic/suppressor lymphocytes. The antigen is also expressed on some Leu-11+ (CD16) cytotoxic NK cells, on a subpopulation of Leu-7+ (HNK-1) cells (which do not have cytotoxic and NK activity), on some Leu-8+ cells (which participate in suppression of B-cell function), and on Leu-15+ (CD11b) cells which are associated with suppressor function.

CD11c (MS, Becton-Dickinson): The antigen is present on monocytes and in low density on granulocytes and large granular lymphocytes in peripheral blood. It is also expressed on macrophages in normal lymphoid tissue, on Kupffer cells in liver, and alveolar macrophages in lung tissue.



Fine needle aspiration of metastases.

Usually seven to ten aspirations were taken from each tumour with a 0.1 mm hypodermic needle. No anesthesia was needed. The aspirate was smeared on a glass slide and was allowed to dry in air. At least two smears were then stained for conventional cytomorphology according to the May-Grünwald-Giemsa method. In cases without obvious melanin pigment in tumour cells the diagnosis of melanoma was confirmed with immunostaining for vimentin and protein S-100. Morphological signs of degeneration or necrosis were registered.

Immunological staining of fine needle aspirates.

The slides were air dried and then fixed for 5 minutes in acetone. After drying the slides were washed in phosphate buffered saline (PBS), pH 7.6, and incubated with monoclonal antibodies against CD4, CD8 and CD11c (see above) for 30 minutes. Mouse IgG (Sigma, Stockholm, Sweden) was used as negative control. After washing in PBS, the sections were incubated with rabbit anti-mouse immunoglobulin (Dakopatts Z 259) and incubated for 30 minutes, washed in TBS and incubated with the PAP mouse monoclonal antibody (Dakopatts, P 850) for 30 minutes. After washing in TBS, the slides were treated with Diaminobenzidin, DAB (Sigma, Stockholm, Sweden) in 3% H<sub>2</sub>O<sub>2</sub> for 6 minutes and washed. The slides were counterstained in Mayers haematoxylin for 15 minutes, washed and mounted in Glycergel (Dakopatts, Sweden). All incubations were performed in a moist chamber.

Preparation of tumour biopsies and immunological staining of tissue sections.

Biopsies from resectable tumours were immediately snap frozen and stored at -70 °C until further processed. Frozen tissue sections, 6-7 µm thick, were fixed in acetone for 10 minutes and then air dried. They were washed in Tris-buffered saline (TBS), pH 7.6 for 5 min, incubated

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with primary antibodies CD4, CD8 and CD11c (see above) for 30 minutes and then washed in TBS for 5 minutes. Mouse IgG (Sigma, Stockholm, Sweden) was used as negative control. The sections were then incubated with rabbit anti-mouse immunoglobulin (Dakopatts, Z 259) for 30 minutes, washed in TBS and incubated with the APAAP mouse monoclonal antibody (Dakopatts D 651) for 30 minutes. After washing in TBS and incubating with the alkaline phosphatase substrate (Naphthol AS-MX Phosphate 2mg (Sigma N4875), Dimethylformamide 0.2 ml, 0.1 M Tris buffer pH 8.2 9.8 ml, 1 M Levamisole 50µl (Sigma L-9756) and Fast-Red TR salt 10mg (Sigma F 1500) for 20 minutes, the sections were washed again in TBS. They were then counterstained in Mayers haematoxylin for 15 minutes and mounted in Glycergel (Dakopatts, Sweden). All incubations were performed in a moist chamber.

#### Evaluation of mononuclear cells.

Because of the often heterogenous distribution of infiltrating inflammatory cells, counting of cells per microscopic field was not performed. The overall occurrence of each subset of these cells, in relation to the number of tumour cells, was instead scored as - (absent), + (sparse, low numbers), ++ (moderate), +++ (high) independently by two investigators. CD4+ cells scored as lymphocytes had small nuclei and sparse cytoplasm with distinct cell membrane. In contrast, CD4+ macrophages displayed large nuclei and abundant, generally faintly staining cytoplasm.

#### Criteria of tumour regression.

Based on the description of regression in primary melanoma in other studies (Mc Govern et al., Kang et al., Sondergaard et al., Ronan et al.) the following criteria of tumour regression were used in this study : 1. Low and variable density of tumour cells, particularly variation in density within the same tumour nodule, 2. Disorganisation of the architecture of the tumour with nests of remaining tumour cells surrounded by stromal tissue. The occurrence of

regressive areas varies from no signs of regression to almost complete destruction of the tumour with only few remaining tumour cells.

The occurrence of regressive changes of the tumours used in this study of patients with resected metastases were estimated to be less than 25% (slight regression), 25-75% and more than 75 % (marked regression).

#### Statistical methods.

The difference in distribution of inflammatory cells between patients with tumour response and progressive disease was analysed using the chi-square test.

#### EXAMPLE 1.

#### Treatment efficacy.

Two groups of patients were studied, those with resectable, regional metastases and those with general metastases. In the latter group the effect of IFN- $\alpha$  treatment was determined as reduction in tumour size. As the main objective of this study was to analyse the importance of tumour infiltrating inflammatory cells for the anti-tumour effect of IFN- $\alpha$ , significant tumour regression (more than 25%) in patients with mixed responses and regressions not fulfilling the criteria for partial remission were registered and used in the following analyses. Among patients with general metastases 11 out of 21 patients had reduction in tumour size. One had a complete remission, 4 partial remission, 3 had a reduction of measurable tumours between 25-50 %, 3 had a mixed response, and 10 patients had progressive disease.

Patients with regional, resectable metastases were treated with IFN- $\alpha$  for only one to three weeks due to the importance of not delaying surgery. This is of course too short a time to allow an adequate determination of the treatment efficacy based on measurement of the tumour size only, thus the determination of therapeutic effect in this group was based on the occurrence of tumour regression in

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the histopathological examination of the tumour biopsies. Metastases with ongoing regression were generally permeated by CD4+ and CD8+ lymphocytes as well as CD11c+ macrophages.

5 Histopathological criteria for signs of spontaneous regression of malignant melanoma has been described in primary tumours. Based on such criteria (described above) nine patients with regional metastases had marked histopathological regression of the resected metastases and 11 patients  
10 had only slight regressive changes.

As no differences regarding tumour regression - determined as reduction in size or occurrence of histopathological regressive changes - were found between the pilot study and the confirmatory study the patients of these studies  
15 were combined in the analyses below. The results obtained in the few (five) patients treated with IFN- $\alpha$  for one week did not seem to differ from the results after 2-3 weeks of treatment, thus, all patients were combined in the following analyses.

20 Subsets of tumour infiltrating mononuclear cells (MNC).

The fine needle aspirates were obtained from lymphnode, subcutaneous and liver metastases. The occurrence of tumour infiltrating mononuclear cells in these aspirates  
25 were studied before initiation of IFN- $\alpha$  treatment. The number and distribution of various subsets of mononuclear cells in the aspirates from the different metastases showed considerable individual variation (Table 1).

30 Table 1.

Occurrence of tumour infiltrating CD4+, CD8+ and CD11c+ inflammatory cells in fine needle aspirates from patients with regional and systemic disease before initiation of IFN- $\alpha$  treatment.

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Number of infiltrating cells	CD4			CD8			CD11c		
	Low	Moderate	High	Low	Moderate	High	Low	Moderate	High
Regional di- sease	6	4	10	9	7	3	6	8	1
Systemic di- sease	10	7	4	14	4	2	6	4	5

Before treatment high numbers of CD4+ cells were found in metastases from 10 out of 20 patients with regional metastases. In all patients with high numbers of CD4+ cells more than 50 % of these cells showed morphologic characteristics of lymphocytes. In contrast, in tumours with low numbers of these cells, CD4+ macrophages were more abundant than CD4+ lymphocytes in 2 out of 6 patients. In patients with moderate numbers of CD4+ cells macrophages were more abundant than lymphocytes in 2 out of 4 patients

In patients with general metastases high numbers of CD4+ cells were found in 4 out of 21 patients before treatment, a result which is significantly different from the frequency found in patients with regional disease ( $P < 0.05$ ). In all of these patients more than 50 % of the CD4+ cells showed morphologic characteristics of lymphocytes. In aspirates from patients with low numbers of CD4+ cells macrophages were more abundant than lymphocytes in 1 out of 10 patients. In patients with moderate numbers of CD4+ cells macrophages were shown to be more abundant than lymphocytes in 1 out of 7 patients.

In patients with regional disease CD4+ cells predominated over CD8+ cells in 7 out of 19 patients. In 10 patients there were no predominance of either subsets and in 2 CD8+ cells predominated over CD4+ cells.

High numbers of CD11c+ macrophages were found in 4 out of 19 patients with regional disease before treatment.

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In the group with general disease high numbers were found in 5 out of 20 tumours.

The occurrence of inflammatory cells in fine needle aspirates and in biopsies after IFN- $\alpha$  treatment were compared in 20 patients. Even if IFN- $\alpha$  might influence the recruitment of these cells there was in general a very good correlation. Lower numbers of CD4+ cells in tissue sections compared to aspirates were found in only 3 out of 20 patients. One of these 3 patients was a responder. All biopsies contained varying degrees of remnants of normal lymph node, which might explain this discrepancy. Alternatively a down regulation might have occurred during IFN- $\alpha$  treatment. Two biopsies showed an increase of CD4+ cells compared to aspirates, one of them was a responder. The size of lymph node metastases seems to be of some guidance as to the presence of remaining normal lymph node. Remnants of normal lymph node was found in 7 out of 13 lymph node metastases less than 20 mm in diameter in contrast to only 2 out of 7 in lymph node metastases of larger size.

#### Correlation to response to IFN- $\alpha$ treatment.

The occurrence of inflammatory cells in relation to the therapeutic effect of IFN- $\alpha$  is shown in Table 2 for patients with general metastases and in Table 3 for patients with regional, resectable metastases. Obviously, there is a very good correlation between anti-tumour effect and the presence of CD4+ lymphocytes in both these groups of patients. The occurrence of these cells in aspirates from responding patients with distant metastases differs significantly from that of patients with progressive tumours ( $p = 0.007$ ). A similar difference is found also for patients with regional metastases, with more CD4+ lymphocytes in metastases showing marked histopathological regressive changes after IFN- $\alpha$  treatment compared to patients with only slight or no regressive changes in the metastases ( $p = 0.077$ ). Surprisingly, there was no correlation between the

occurrence of CD8+ or CD11c+ cells and response to IFN- $\alpha$  treatment, neither in regional nor in general disease.

Table 2.

5 Number of tumour infiltrating CD4+, CD8+ and CD11c+ inflammatory cells in patients with inoperable systemic metastatic disease according to clinical effect of IFN- $\alpha$  treatment.

Number of infiltrating cells	CD4 Therapeutic effect			CD8 Therapeutic effect			CD11c Therapeutic effect		
	R	MR	PD	R	MR	PD	R	MR	PD
Low	1	0	9	4	2	8	3	1	3
Moderate	4	2	1	1	1	2	3	1	4
High	3	1	0	2	0	0	1	1	3

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Table 3.

Number of tumour infiltrating CD4+, CD8+ and CD11c+ inflammatory cells in patients with regional disease according to histopathological regression after IFN- $\alpha$  treatment.

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Number of infiltrating cells	CD4		CD8		M5	
	Marked histopat.	Slight regr.	Marked histopat.	Slight regr.	Marked histopat.	Slight regr.
Low	1	5	3	6	2	4
Moderate	1	3	3	4	4	4
High	7	3	2	1	2	3

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According to the present invention a good correlation was obtained between the occurrence of a specific class of cells and the therapeutic response to a drug against a tumour. In the example given this correlation was obtained

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with the helper T subset and the therapeutic benefit of IFN- $\alpha$ . In patients with regional disease 7 out of 10 patients with high numbers of these cells in fine needle aspirates achieved marked histopathological tumour regression. In contrast, in patients with low numbers of cells of this subset 5 out of 6 patients achieved only a slight regression of their tumours. In the group with distant metastases 10 out of 11 of those with moderate or high infiltration of cells of this subset in the tumours achieved tumour regression. In contrast, among patients with low infiltration of these cells 9 out of 10 had progressive disease. Based on these results there seems to be a need for cells of this subset infiltrating the tumours before start of treatment with IFN- $\alpha$  to make the treatment successful. Thus, the degree of infiltration of these cells seems to be a useful predictive test for choosing patients suitable for this therapy.

In order to simplify and facilitate the diagnostic techniques with a fine needle aspirate of a tumour from a patient the use of a diagnostic kit is applied. An example of such a kit is given below.

A single cell suspension the fine needle aspirate is prepared by suspending the aspirate in a solution containing a mixture of the enzymes collagenase, hyaluronidase and DNAase. A mixture of monoclonal antibodies directed towards tumour associated antigens on tumour cells and labelled with one type of fluorochrome (type A) is added to the cell suspension thus obtained. Then a mixture of monoclonal antibodies directed towards CD3 (a T-lymphocyte marker) and CD4 antigens on lymphocytes is added, i.e. anti-CD3 antibodies labelled with a fluorochrome type B and anti-CD4 antibodies labelled with a fluorochrome type C. Such a double staining is used in order to identify CD4+ lymphocytes and avoid the counting of CD4+ monocytes/macrophages. The numbers of tumour cells and double stained



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lymphocytes are then determined by means of flow cytometry.  
A high ratio CD4+ lymphocytes/tumour cells will according  
to the invention identify responders to immunotherapy.

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## CLAIMS

1. Method of predicting the therapeutic response of a drug against a malignant tumour in a human patient, using a sample of a fine needle aspirate of said tumour from said patient, characterized in that the method comprises the steps of identifying and estimating in said sample the number of cells belonging to one class of cells in relation to other cells in said sample; and comparing said estimation with a previously made analysis demonstrating correlation between the number of said cells and parameters of the therapeutic response of said drug directed towards said tumour in human patients; the results of said comparison being indicative of the therapeutic response of said drug in said human patient.
2. Method as in claim 1, characterized in that said drug is an immunomodulating drug.
3. Method as in claim 1, characterized in that said class of cells is a subset of tumour infiltrating mononuclear cells.
4. Method as in claim 3, characterized in that said subset of tumour infiltrating mononuclear cells is the helper T subset.
5. Method as in claim 1, characterized in that said tumour is a metastatic malignant melanoma and said drug is interferon-alfa.
6. Method as in claim 1, characterized in that said cells are identified and estimated by means of immunological staining.
7. Method as in claim 6, characterized in that said immunological staining is performed with monoklonal antibodies.

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8. Diagnostic kit useful for identifying responders to immunotherapy when predicting the therapeutic response of a drug directed towards a malignant tumour in a human patient, characterized in that said kit  
5 comprises  
a mixture of enzymes rendering a sample of a fine needle aspirate of said tumour from said patient a single cell suspension,  
a first mixture of monoclonal antibodies directed towards  
10 tumour associated antigens on tumour cells in said single cell suspension, said antibodies having a first label, and  
a second mixture of two types of monoclonal antibodies directed towards two specific antigens on lymphocytes, said two types of antibodies having a second and a third label,  
15 respectively,  
the numbers of tumour cells and double stained lymphocytes being prepared for determination by blood counting.
9. Diagnostic kit as in claim 8, characterized -  
i z e d in that said mixture of enzymes comprises  
20 collagenase, hyaluronidase and DNAase.
10. Diagnostic kit as in claim 8, characterized -  
i z e d in that said two specific antigens on lymphocytes are CD3 and CD4 antigens.
11. Diagnostic kit as in claim 8, characterized -  
25 i z e d in that said first label is a fluorochrome type A, and said second label and said third label is a fluorochrome type B and C, respectively.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02007

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 G01N33/50 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim 1 o.
Y	<p>CANCER IMMUNOL. IMMUNOTHER.,  vol. 37, no. 6, 1993,  pages 367-372, XP000603158  A. EISENTHAL ET AL.: "Phenotypic and  functional profile of peripheral blood  mononuclear cells isolated from melanoma  patients undergoing combined immunotherapy  and chemotherapy."  see the whole document</p> <p>---  -/--</p>	1-11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

25 September 1996

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Cartagena y Abella;P

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02007

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	--- THE JOURNAL OF IMMUNOLOGY, vol. 136, no. 5, 1 March 1986, pages 1899-1907, XP002014260 S. MIESCHER ET AL.: "Functional properties of tumor-infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes." cited in the application see page 1899 - page 1900	8,9
A	--- WO,A,92 08981 (T CELL DIAGNOSTICS, INC.) 29 May 1992 see claims 41,42	1
A	--- WO,A,88 01385 (J. ANDERSON) 25 February 1988 see claim 9	1
A	--- INTERNATIONAL THERAPY IN EARLY STAGE MELANOMA PATIENTS, vol. 3, no. 3, 1 January 1995, pages 265-272, XP000502849 N. STANOJEVIC-BAKIC ET AL.: "T- activin therapy in early stage melanoma patients: in vitro and in vivo immunologic effect." -----	



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Information on patent family members

International Application No

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